

# The development of a new multiplex dipstick for the simultaneous detection of sulfonamides, (fluoro)quinolones, tylosin and chloramphenicol in honey

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[www.confidence.eu](http://www.confidence.eu)



# CONFIDENCE project

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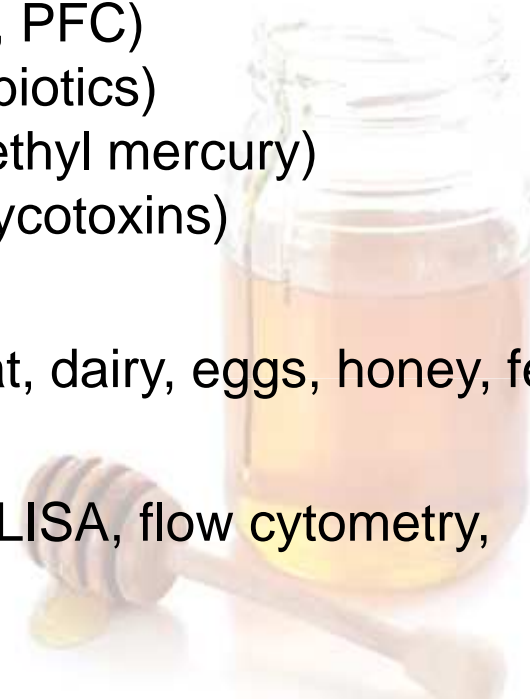
- **CONTaminants in Food and Feed : Inexpensive DETection for Control of Exposure...**
- **Collaborative Project** : FP7 - European Commission
- **Duration**: 4 years (May 2008 – April 2012)
- **Partners**: 16 partners from 10 countries (universities, SME, research institutes,...)
- **Budget**: 7.5 Mio €
- **Coordinator**: RIKILT - Institute of Food Safety (NL)
- **Objective**: Development of innovative, reliable, simple, fast and multiple screening tests for chemical contaminants and residues in food and feed



# CONFIDENCE project

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- **Target analytes:**
  - Organic pollutants (PCB, BFR, PAH, PFC)
  - Veterinary drugs (coccidiostats, antibiotics)
  - Heavy metals (inorganic arsenic, methyl mercury)
  - Biotoxins (alkaloids, phycotoxins, mycotoxins)
- **Commodities:** seafood, cereals, meat, dairy, eggs, honey, feed
- **Techniques:** dipsticks, biosensors, ELISA, flow cytometry, cytosensors, simplified GC/LC-MS
- **Final goals:**
  - Delivering tools to improve food safety
  - Enable more frequent testing
  - Shift testing to the start of the supply chain



# CONFIDENCE for honey

## ➤ Antibiotics

- *Electrochemical immunosensor*  
12 sulfonamides < 25 µg/kg

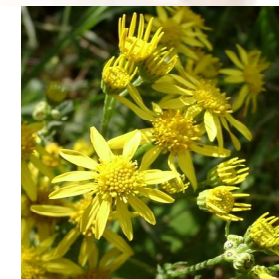
- *Multiplex dipstick*

sulfonamides, tylosin, quinolones, chloramphenicol

## ➤ Pyrrolizidine alkaloids (PA)

- original plan: multiplex dipstick for lycopsamine + jacobine  
- major difficulties in dipstick format  
- revision of scope of analytes

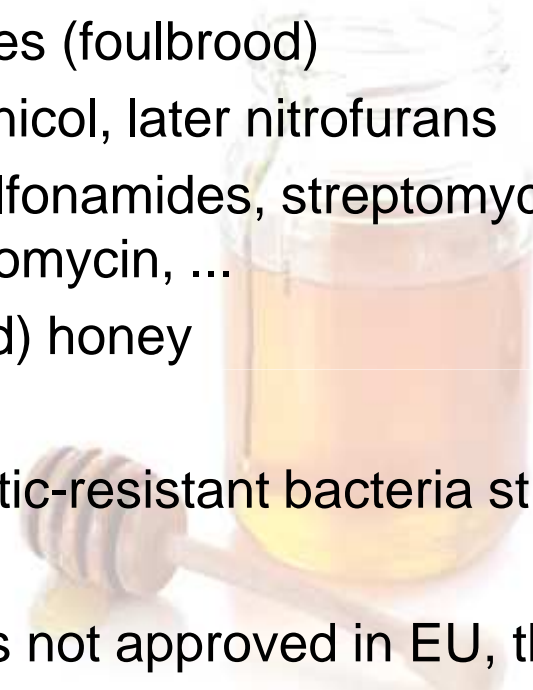
➔ currently preparation of new antibodies,  
shifting to ELISA format



# Antibiotics in Honey



- Use of antibiotics by some beekeepers to cure or prevent bacterial infestations of hives (foulbrood)
- 2002/03 alerts relating to chloramphenicol, later nitrofurans
- Continuous usage of tetracyclines, sulfonamides, streptomycin, tylosin, quinolones, lincomycin, erythromycin, ...
- Multiple antibiotics present in (blended) honey
- Concerns about emergence of antibiotic-resistant bacteria strains
- The use of antibiotics in beekeeping is not approved in EU, thus absence is required
- Other countries handle Maximum Residue Limits
- Testing required in both cases!



# Multiplex assay concept

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To develop, validate and demonstrate the impact of novel **multiplex dipsticks** for the **rapid, easy** and **cost-effective** detection of the presence of some frequently detected **antibiotics in honey** including...

Sulfonamides

Chloramphenicol

Tylosin

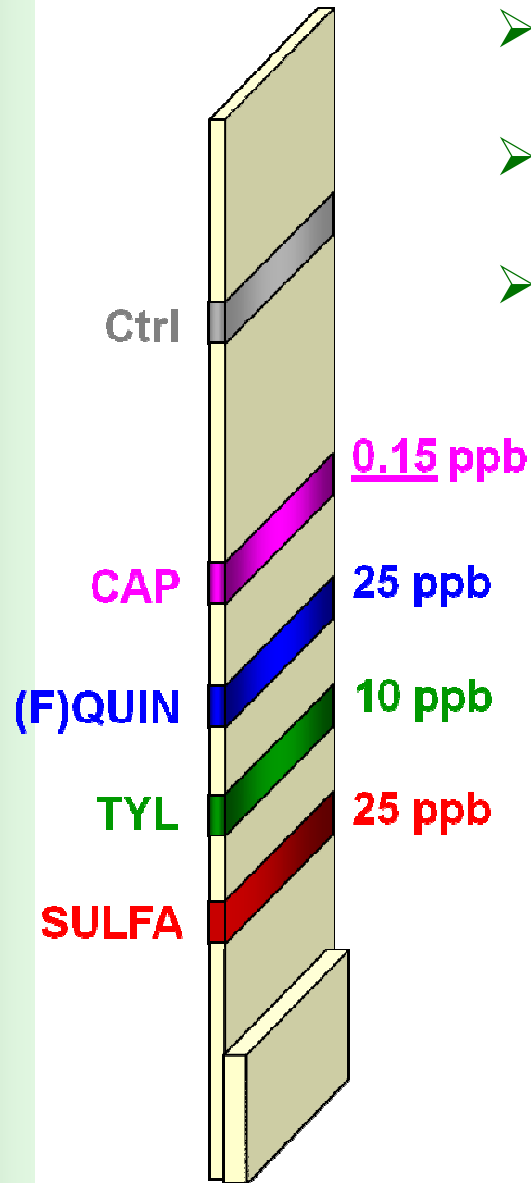
(Fluoro)quinolones



*(dipstick test available for tetracyclines)*



# Multiplex assay concept



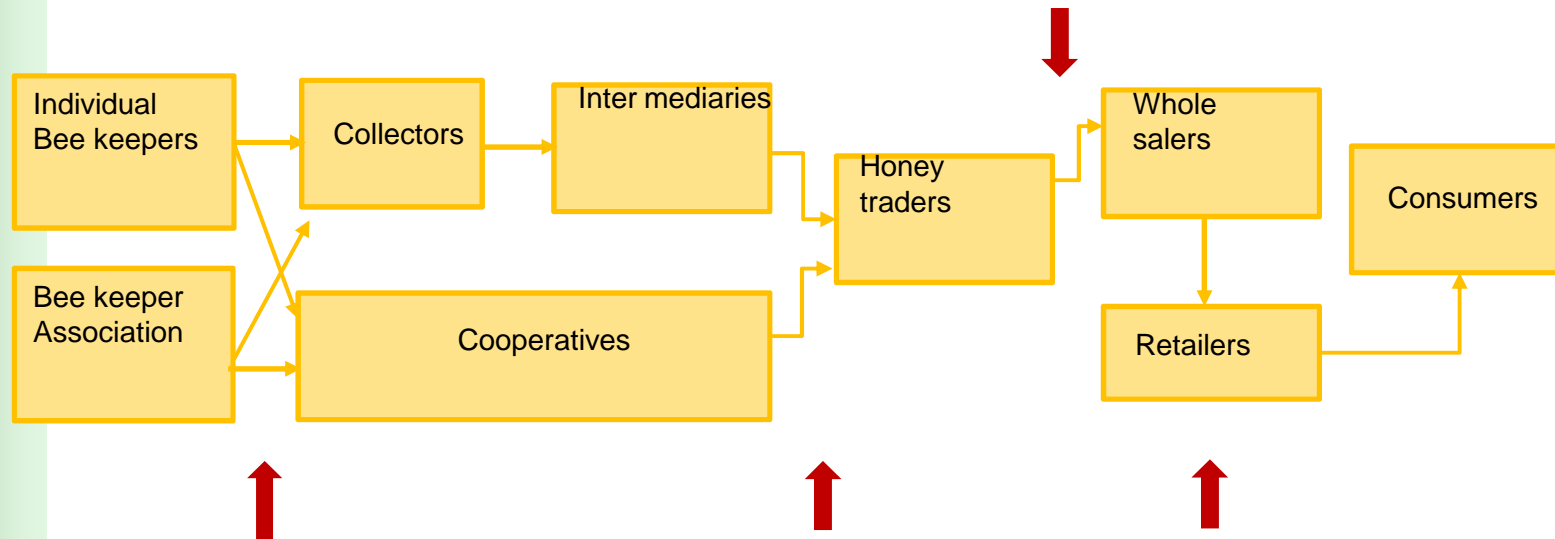
- Competitive inhibition format (Lateral flow device);
- Incorporating 4 test lines and 1 control line;
- Exploiting matched pairs of antibodies and analyte-protein (OVA) competitors;



# Assay formats

## ➤ Lab-based assay

- simple extraction
- sensitive, meets recommended reference levels (for sulfonamides, tylosin, quinolones)
- suited for honey sector QC/QA labs along supply chain as well as external contract labs

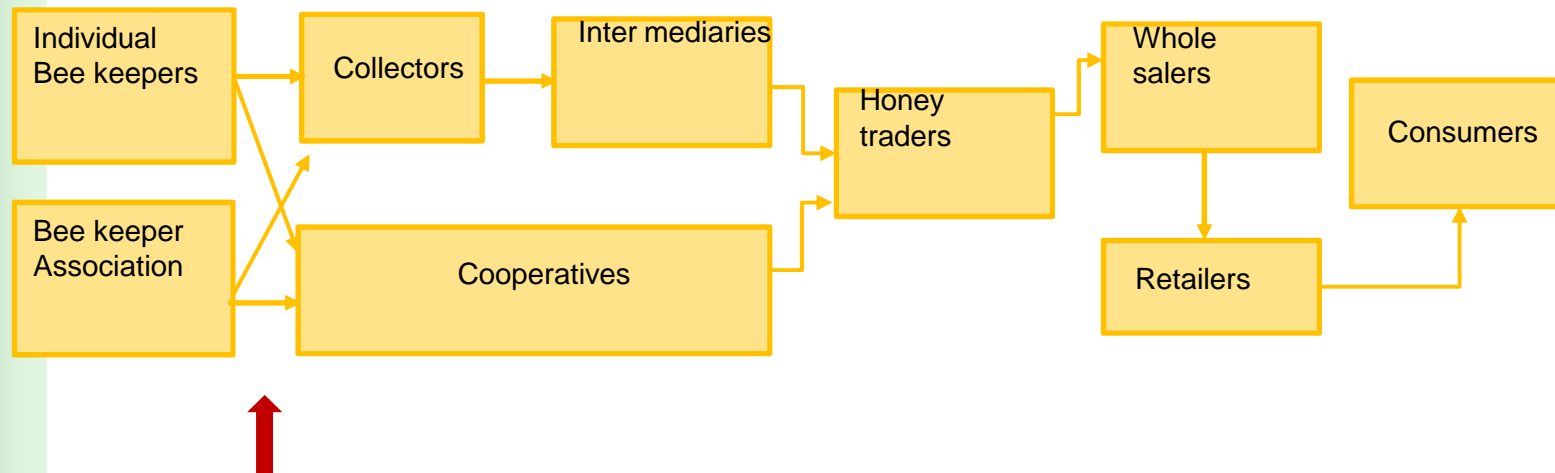




# Assay formats

## ➤ Field assay

- no lab equipment required, no extraction
- less sensitive than lab assay, but sufficient to detect contaminated batches from treated hives
- suited for collectors, cooperatives to test individual lots from beekeepers



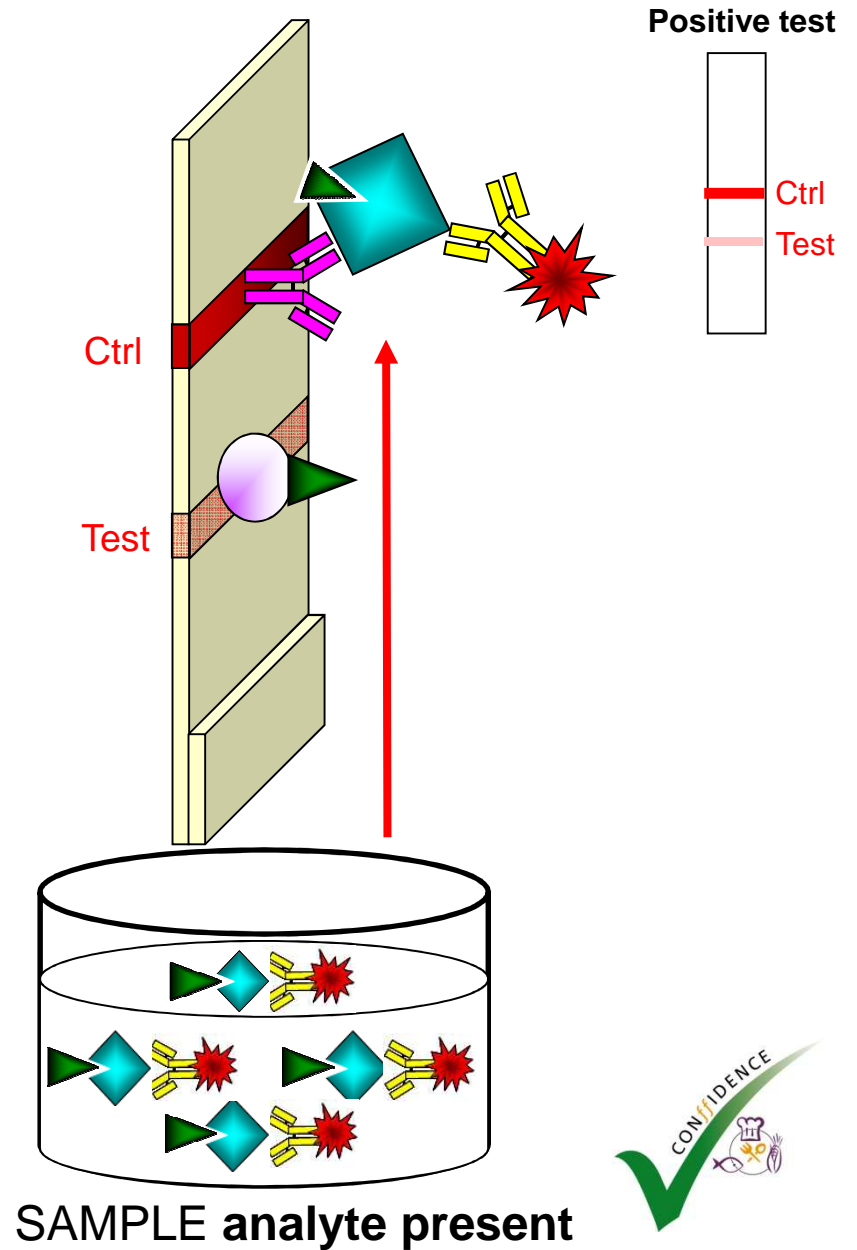
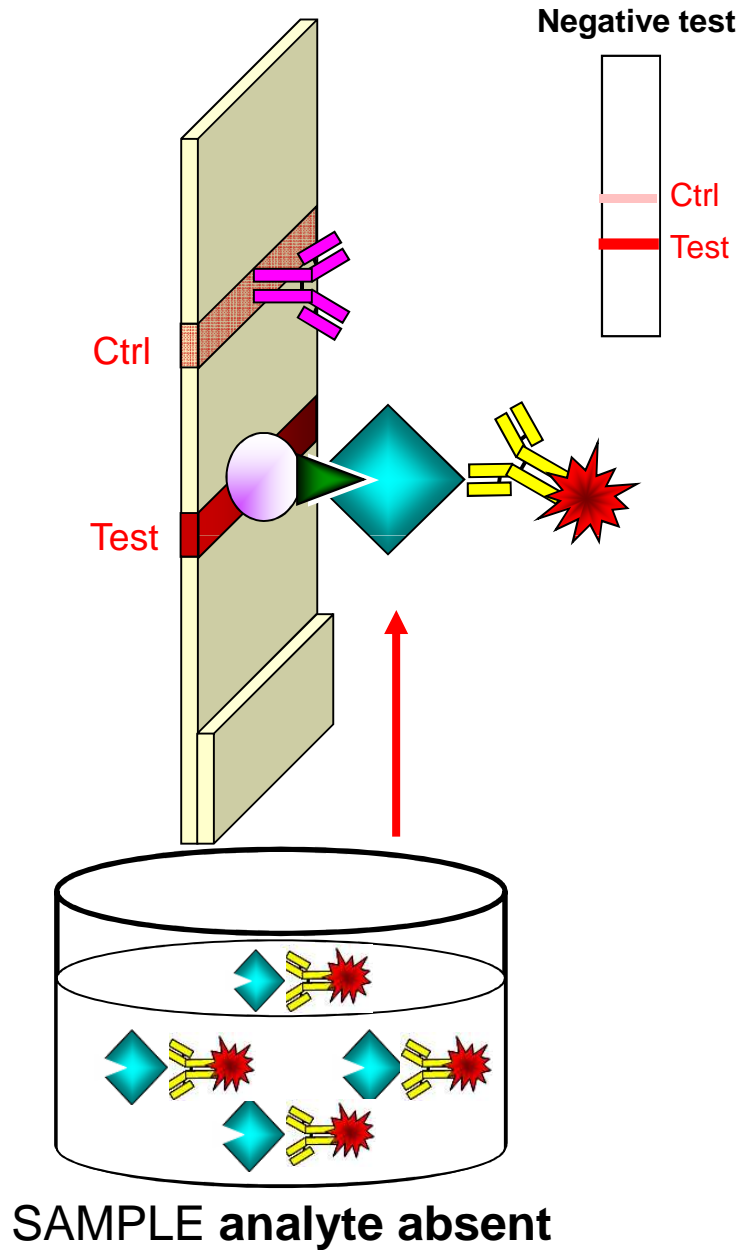
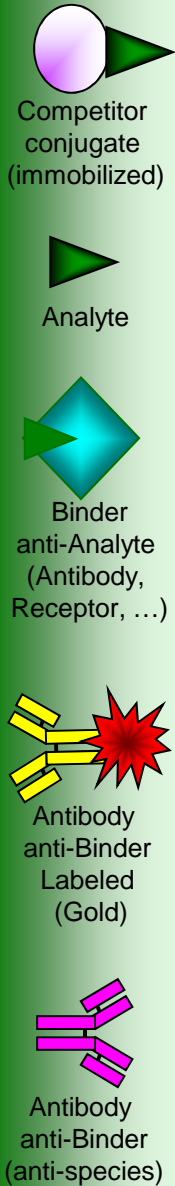
# Field study

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- Evaluation of the applicability and performance of the assay under field conditions
- Testing honey from routine flow (+control samples) with supplied test kit
- Interested cooperatives, collectors, aggregators can register via website or e-mail:
  - [www.confidence.eu](http://www.confidence.eu)
  - [dipstick@confidence.eu](mailto:dipstick@confidence.eu)



# Indirect competitive dipstick principle



# Challenge for a multiple test in honey

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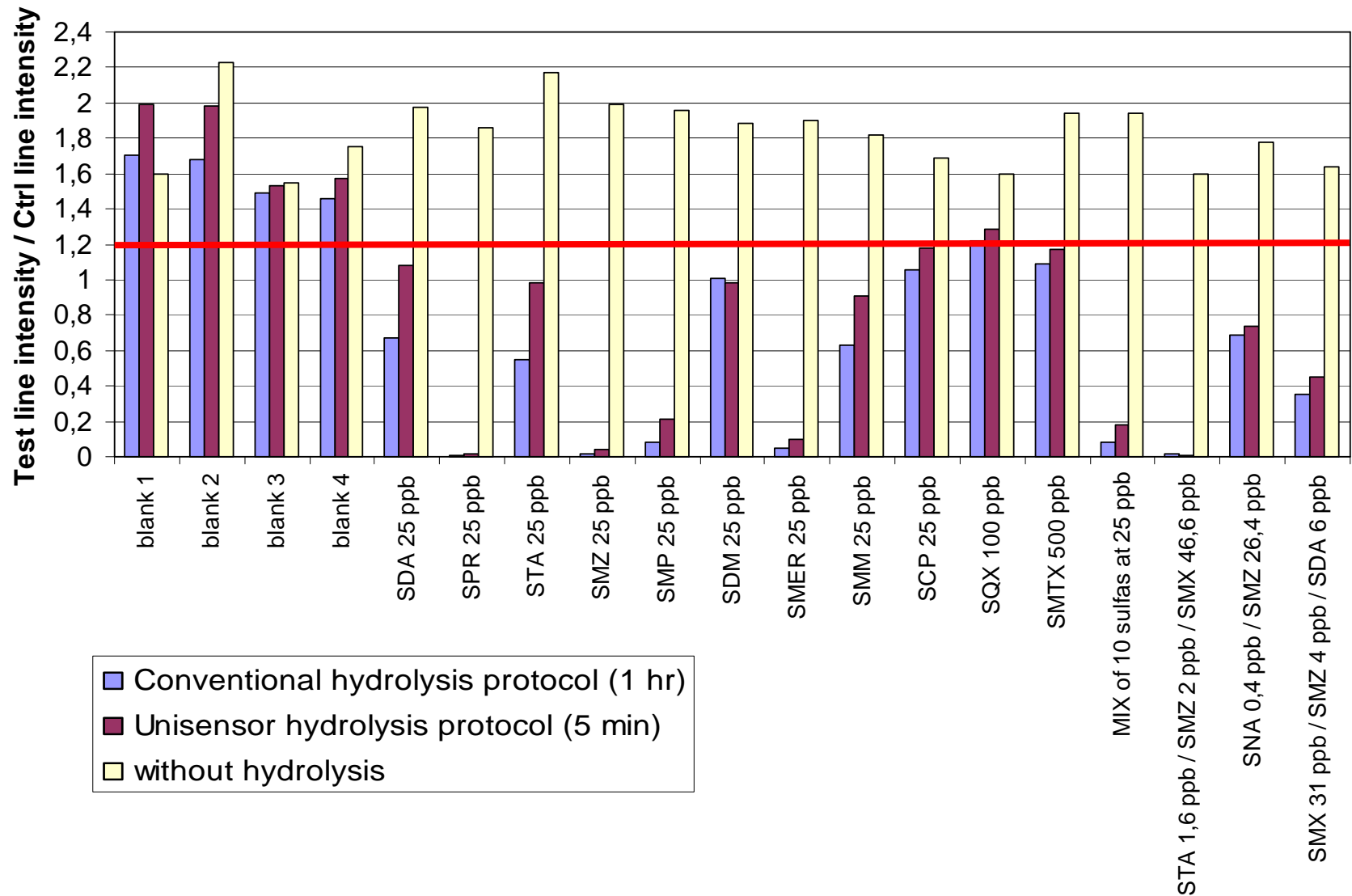
- Binding of **SULFAMIDES** to reductive sugars of honey and **QUINOLONES** better soluble in acidic conditions...

→ *NEED of an **acidic hydrolysis** of the sample for drug release/solubilization...*



# Challenge of a multiple test for honey

- Development of an easy/rapid hydrolysis for sulfa release...



# Challenge for a multiple test in honey

- Binding of **SULFAMIDES** to reductive sugars of honey and **QUINOLONES** better soluble in acidic conditions...

→ *NEED of an **acidic hydrolysis** of the sample for drug release/solubilization...*

## BUT...

- **TYLOSIN** degrades in acidic condition and **CHLORAMPHENICOL** has a MRPL at 0.3 µg/kg in honey...

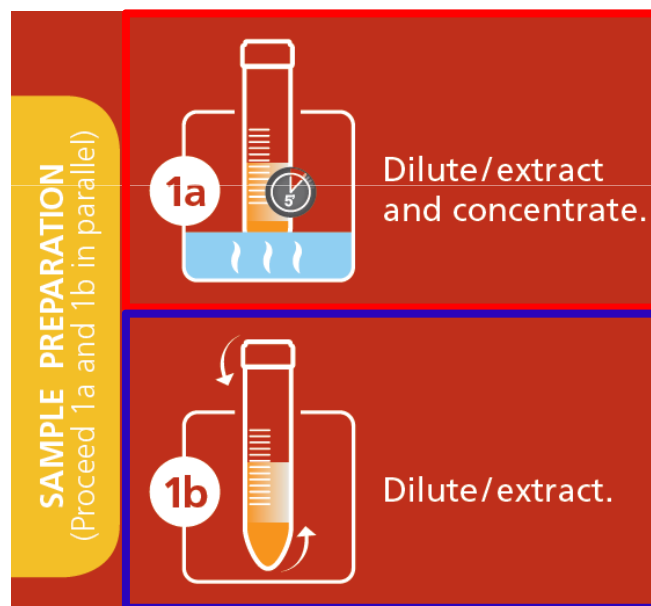
→ *NEED to **avoid acidic** condition and to use of **solvent** extraction/concentration to reach high sensitivity...*



# Challenge of a multiple test for honey

## SOLUTION:

- 2 separate honey samples diluted in parallel...
- Pool of the 2 samples just before dipstick analysis



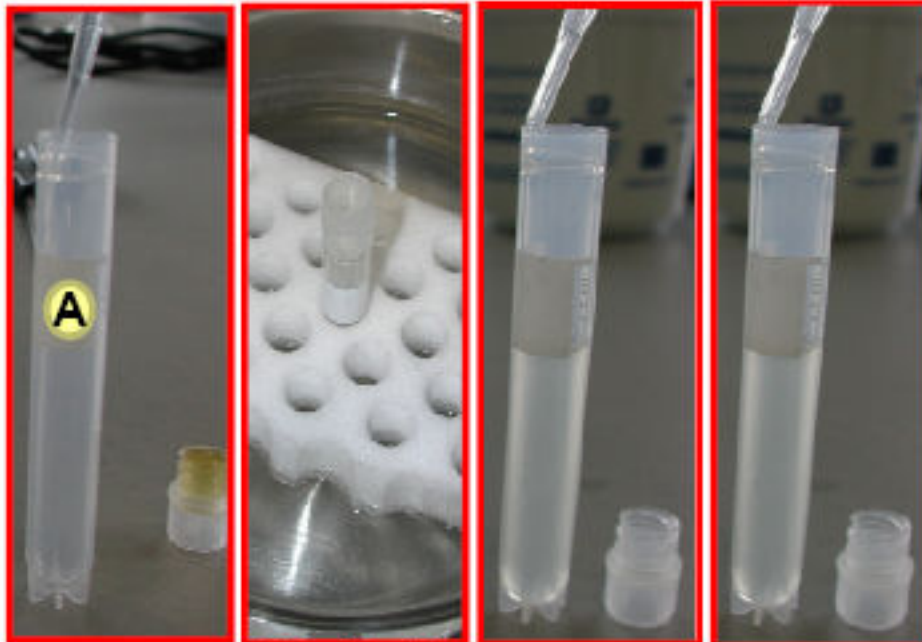
Acidic **hydrolysis** (SULFA / QUINO release)

Buffer **dilution** (TYL / CAP protection)



# Field-test test : method schematic

## 1. DILUTION / HYDROLYSIS

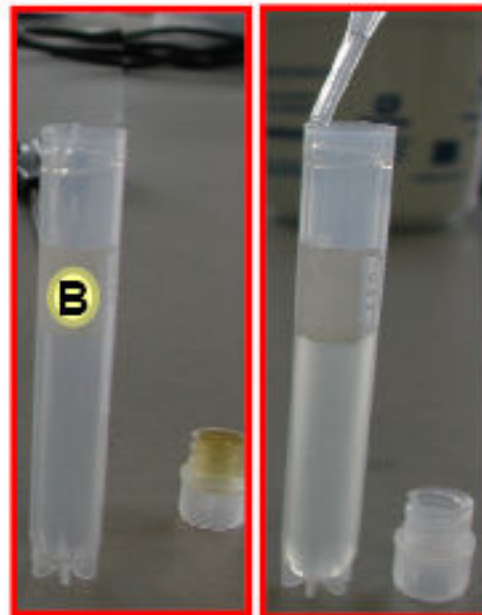


0,65 gr HONEY

300  $\mu$ l Acid Hydrolysis (5 min 95°C)

300  $\mu$ l Base Neutralization

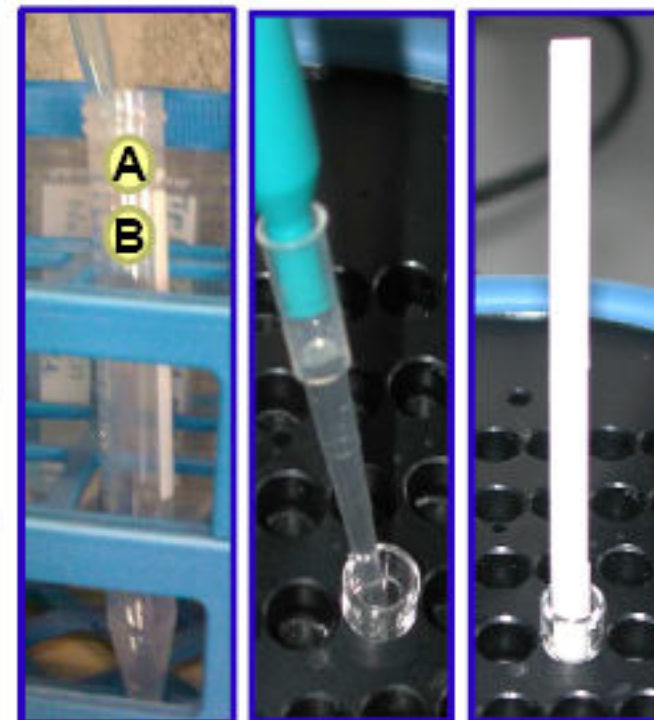
2,4 ml buffer Dissolution



0,65 gr HONEY

3 ml buffer Dissolution

## 2. DIPSTICK



Mix A & B 200 $\mu$ l/200 $\mu$ l

5 min Incubation at 25°C (RT)

15 min Dipstick At 25°C (RT)

**<30 min  
TOTAL**

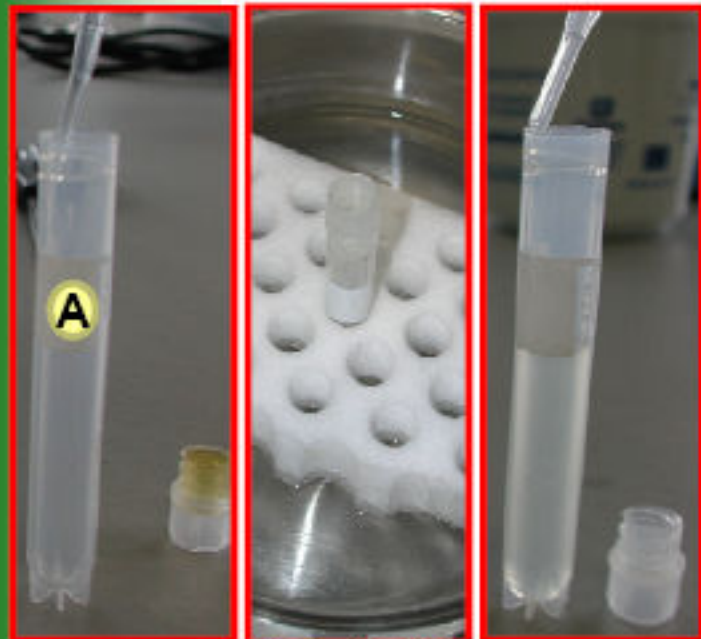
**All material  
provided in  
the kit !**





# Lab-test format : method schematic

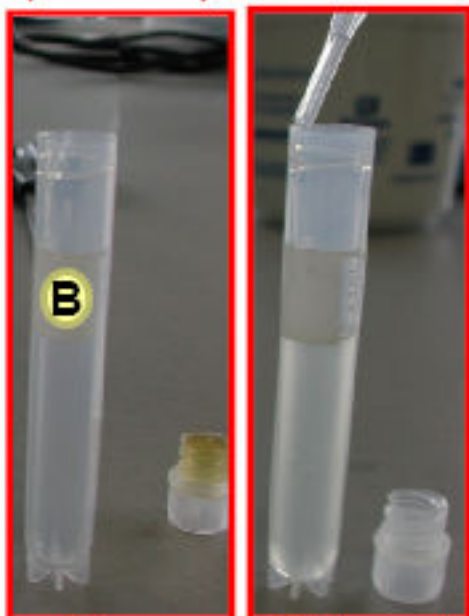
## 1. DILUTION / HYDROLYSIS



2,5 gr  
HONEY

1200  $\mu$ l Acid  
Hydrolysis  
(5 min 95°C)

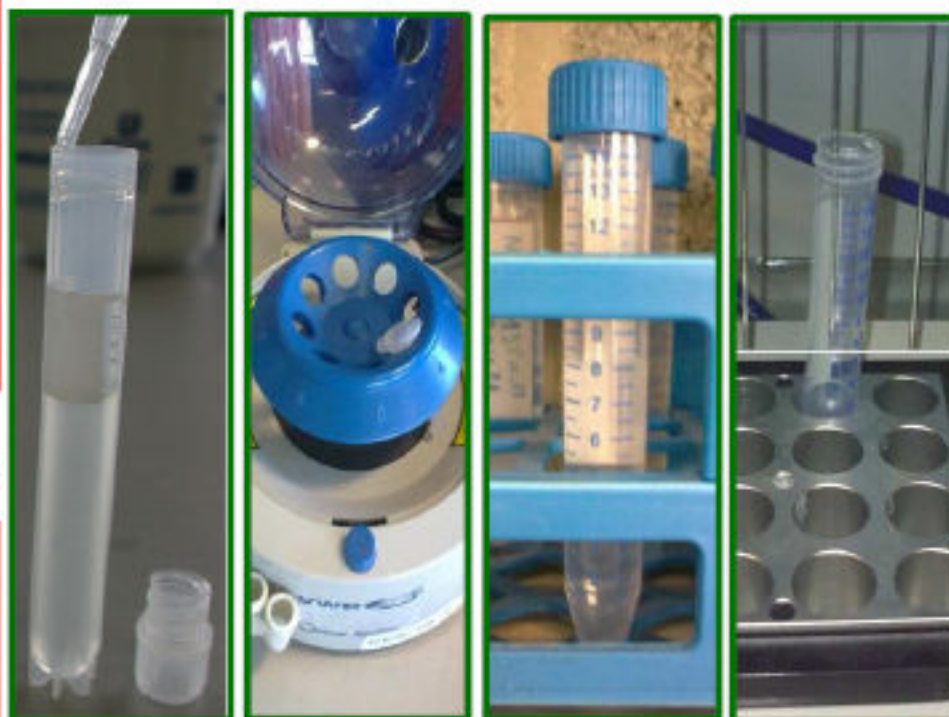
1200  $\mu$ l Base  
Neutralization



2,5 gr  
HONEY

2400  $\mu$ l warm H<sub>2</sub>O  
For dissolution

## 2. EXTRACTION



10 ml EA  
shake  
(10min)

Centrifuge  
(5 min)

Transfer 8ml  
Supernatant

Evaporation  
(40 min 55°C)

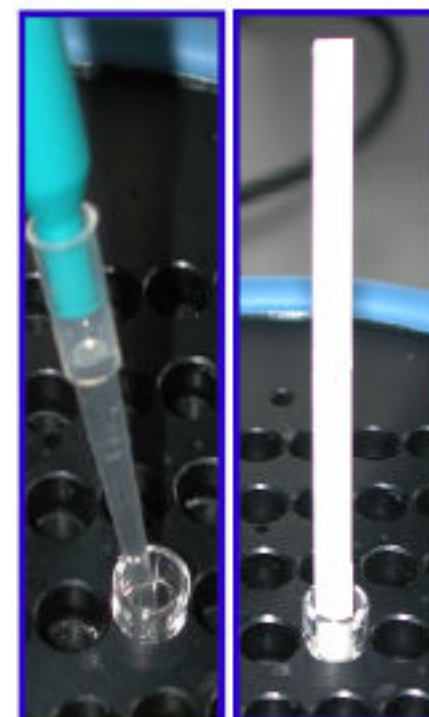
## 3. DIPSTICK



Dilution 250 $\mu$ l  
Buffer –  
Mix 200 $\mu$ l A&B



Dilution 250 $\mu$ l  
Buffer –  
Mix 200 $\mu$ l A&B



5 min  
Incubation  
40°C

15 min  
Dipstick  
40°C



# Optional tools for the dipstick analysis



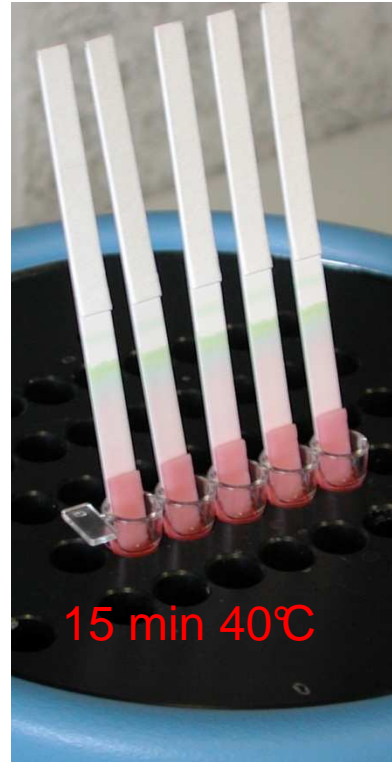
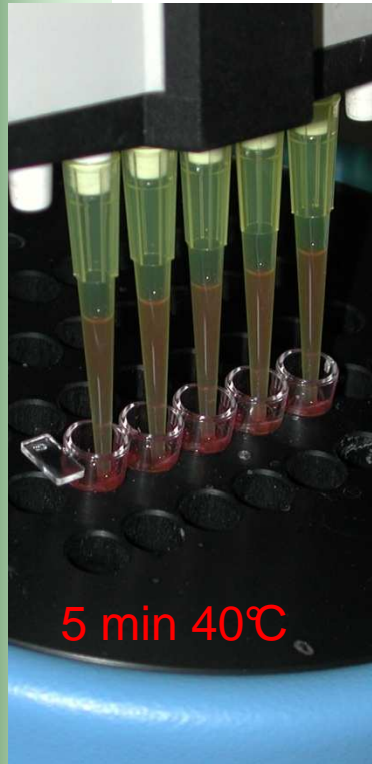
**Heatsensor®** makes the dipstick analysis automatic in one single step...



**Readsensor®** makes dipstick measurement more objective, traceable and semi-quantitative

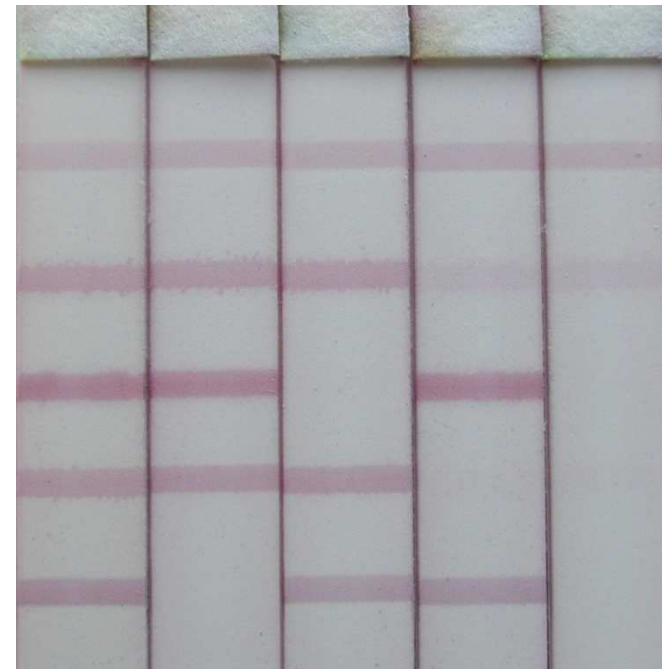


# Lab test multiplex dipstick results



BLANK HONEY  
 SULFA 25 ppb\*  
 QUINO 25 ppb\*\*  
 TYL 10 ppb/CAP 5 ppb  
 MIX OF ALL \*\*\*

CTRL  
 CAP  
 QUINO  
 TYL-A  
 SULFA



\* Mix of 10 SULFA spiked in honey at a TOTAL concentration of 25 µg/kg (ppb) : SDA, SPR, STA, SMZ, SMP, SDM, SMER, SMM, SCP, SQX.

\*\* Mix of 8 QUINO spiked in honey at a TOTAL concentration of 25 µg/kg (ppb) : CIPRO, DANO, DIFLO, ENRO, FLUM, MARBO, NOR, SARA.

\*\*\* Mix of SULFA / QUINO / TYL-A / CAP spiked in honey at 25 µg/kg / 25 µg/kg / 10 µg/kg / 5 µg/kg (ppb).



# Lab test validation

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## ➤ HONEY SAMPLES USED:

- Liquid, solid, amber, dark, pale, raw, commercial...
- Blank vs Spiked (STA / CIPRO / TYL / CAP at 25 / 25 / 10 / 5 µg/kg)

## ➤ SENSITIVITY:

- **100%** of **positive** results at 1/2 screening target concentrations for Sulfathiazole, Ciprofloxacin, Chloramphenicol
- **90%** of **positive** result at screening target concentration for Tylosin

## ➤ RUGGEDNESS (n=20):

- Temperature for extract evaporation = **50°C +/-5°C**
- Time flexibility to read result = **Directly** but OK after **10 & 20 minutes**
- Potential decrease of Tylosin sensitivity for raw honey containing wax

## ➤ SPECIFICITY (compounds at 50µg/kg) :

- No interference on the test with other antibiotics
- Very slight crossreactivity of FQ line with Fumagillin



# SENSITIVITY ( $\mu\text{g}/\text{kg}$ – ppb)

Sulfonamide compounds	LoD LAB	LoD FIELD	CRL**	(Fluoro)quinolone compounds	LoD LAB	LoD FIELD	CRL**
Sulfapyridine	<10	<50	50	Enrofloxacin	<25	5-25	50
Sulfamethazine	<25	<50		Ciprofloxacin	<25	50	
Sulfamethoxypyridazine	25	50-100		Danofloxacin	25-50	<100	
Sulfamerazine	25	50-100		Difloxacin	250	<500	
Sulfamonomethoxine	25	50-100		Marbofloxacin	50	<100	
Sulfadiazine	25	50-100		Norfloxacin	25	50	
Sulfadimethoxine	25	50-100		Sarafloxacin	>500	-	
Sulfathiazole	25	50-100		Flumequine	>500	-	
Sulfachloropyridazine	25	50-100					
Sulfaquinoxaline	50	<200					
Other compounds	LoD LAB	LoD FIELD	CRL**				
Tylosin-A	10	10-50	10				
Chloramphenicol	5	<60	0.3				

\*\* European limits or recommended concentrations in honey (CRL – AFSSA-LMV France – SANCO /2006/3228).

# Availability of the MULTIPLEX

- Extern Lab Validation in progress (FERA, UK) and Inter Lab Validation in January 2012
- Completing the range of existing UNISENSOR's dipstick assay detecting antibiotics in Honey (Tetracyclines, Sulfamides)
- Kit produced and commercialized by [unisensor](http://www.unisensor.be)  under the name **bee4sensor**



[www.unisensor.be](http://www.unisensor.be)



# Conclusions

- Development of a **multiplex dipstick** assay detecting **antibiotics in honey...**



✓ **Rapid** - Results in 30 (field) or 90 min (lab)



✓ **Multiple** - Detection of more than 18 relevant antibiotics in one single test



✓ **Discriminating** – Direct determination of the antibiotic class in case of positive result



✓ **Flexible** - Flexibility regarding sensitivity, time and material availability



✓ **User-friendly** - Clear visual result or reader interpretation

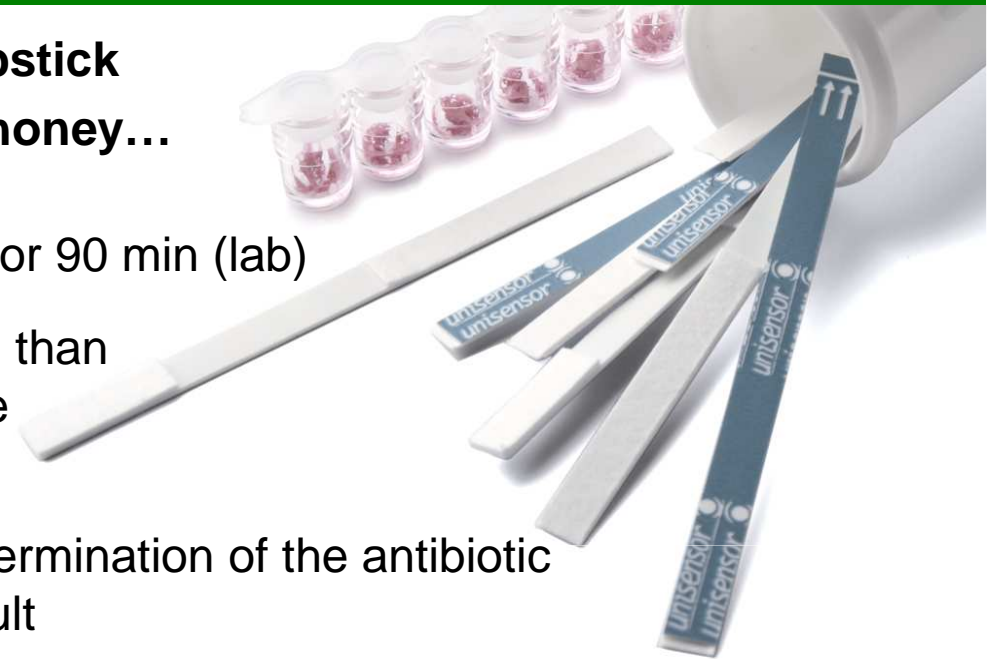


✓ **Convenient** - Performable on site or in the lab



✓ **Reliable and robust**

✓ **Cost-Effective** - Does not need any expensive instrumentation



# Thanks to...

- Multiplex dipstick development :
  - **UNISENSOR S.A.** (Belgium)
  - **CER** (Belgium)
  - **CSIC** (Spain)
  
- Matrix preparation & lab validation :
  - **FERA** (United Kingdom)
  - **NESTLE NRC** (Switzerland)
  
- Project coordination :
  - **RIKILT** (The Netherlands)
  
- Funding :
  - **CONFIDENCE** (European Commission FP7 Grant agreement n°211326)

